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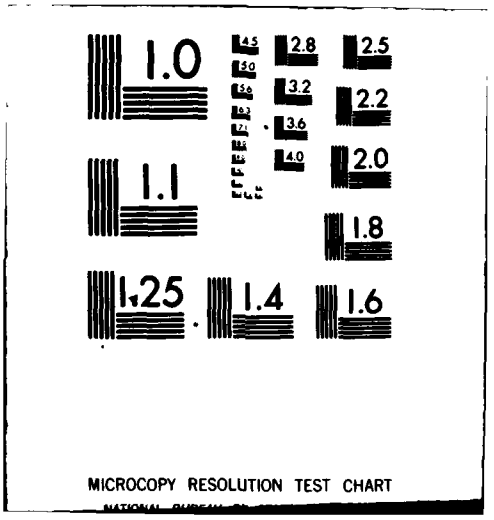
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OFFICE OF NAVAL RESEARCH

Contract #ONR N00014-76-C-0053

Task No. NR 204 - 002

ANNUAL REPORT NO. 4

Penetration Enzymes of Schistosome Cercariae.

by

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and

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BACKGROUND

The infective process during which schistosome cercariae invade the skin of vertebrate hosts includes several aspects about which our knowledge is incomplete. These aspects concern changes in the relationship of the cercariae to the snail host in which they developed, in penetrated vertebrate host skin, and in the invading parasite itself. In order better to understand the infective process, data have been accumulated and analyzed under the following headings: (1) the infective process - fluctuations in snail-cercaria relations; (2) the infective process - the rotifer problem; (3) morphological (fine structural) changes as cercariae transform to schistosomules; and (4) antigenicity of secreted cercarial preacetabular enzyme.

I. THE INFECTIVE PROCESS - FLUCTUATIONS IN SNAIL-CERCARIA RELATIONS

Methodology. Under our conditions, fluctuations in the snail-parasite phase of the life cycle of Schistosoma mansoni in the laboratory are to be expected. The variations have been monitored. Parameters were: average cercarial production per snail; percentage of exposed snails infected; percentage of deaths of snails during prepatency; percentage of deaths of snails during patency. Maintenance conditions were varied to test their influence on the snail-cercaria relationship. Records were kept of performance of snails exposed to 6 to 8 or 8 to 10 miracidia and maintained routinely, cercariae being collected twice weekly.

Results. Great improvement has been made in cercarial production per snail (Fig. 1). This has been gradual as maintenance conditions

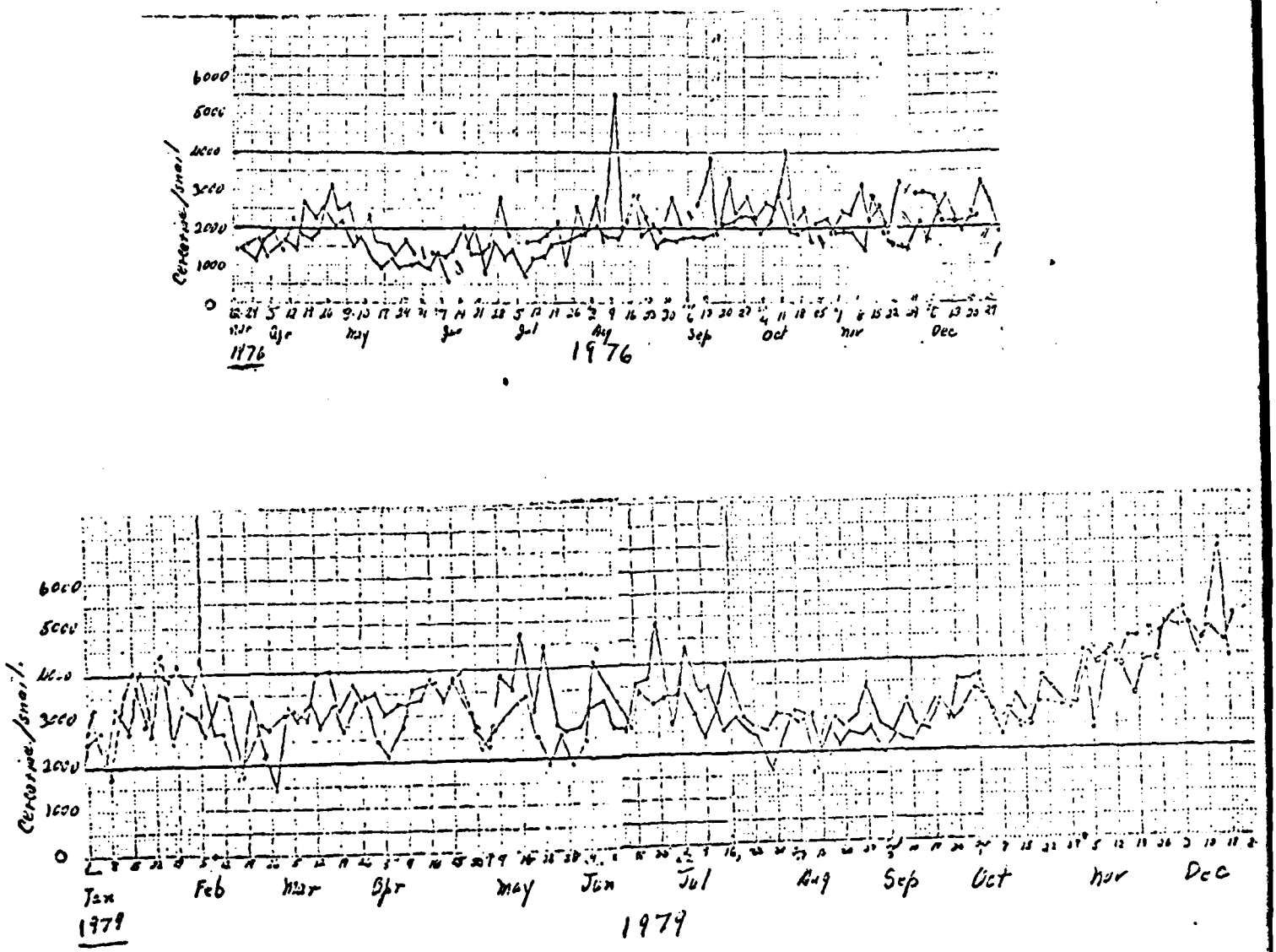

Fig. 1. Comparison of the average number of cercariae collected per snail per day in 1976 and 1979.

were tested and the favorable ones adopted. These data suggest that critical conditions for high cercarial collections were: 1) a warm (26-27 C) dark maintenance environment for exposed and infected snails; 2) a strongly lighted warmer (30-31 C) chamber for harvesting cercariae from infected snails; 3) immediate transfer of snails without delay from the warm dark aquaria in the dark room to cercarial collection beakers in the lighted (30-31 C) collection chamber; 4) elimination, insofar as possible, of symbiotic rotifers from the snails; 5) maintenance of stock snails in a healthy well-fed condition.

The low cercarial output from March to August 1976 reflects the usual spring and early summer depression resulting from cyclic heavy infestation by symbiotic rotifers. This infestation and the resultant depression of cercarial output did not occur in 1979, probably because the snails were washed by jet water spray as soon as any rotifers were noted on their shells thus preventing any heavy rotifer buildup. The slight decline in the average number of cercariae per snail from mid-July to November 1979 was concurrent with decreased intensity of the light from an ageing bulb in the cercarial collection chamber. The old bulb was replaced after the collection on 29 October 1979, and the cercarial output increased.

Cercarial production was not related to the level of miracidial exposure of the snails if the levels at test were 6-8 as compared with 8-10 miracidia. No diminution of cercarial output by the snails was observed after mid-April 1979 at which time the snail exposure level was reduced from 8-10 to 6-8 miracidia per snail.

There were also variations in parameters other than cercarial output (Fig. 2), notably in the percentage of snails which became infected and the percentage of snail deaths during the prepatent

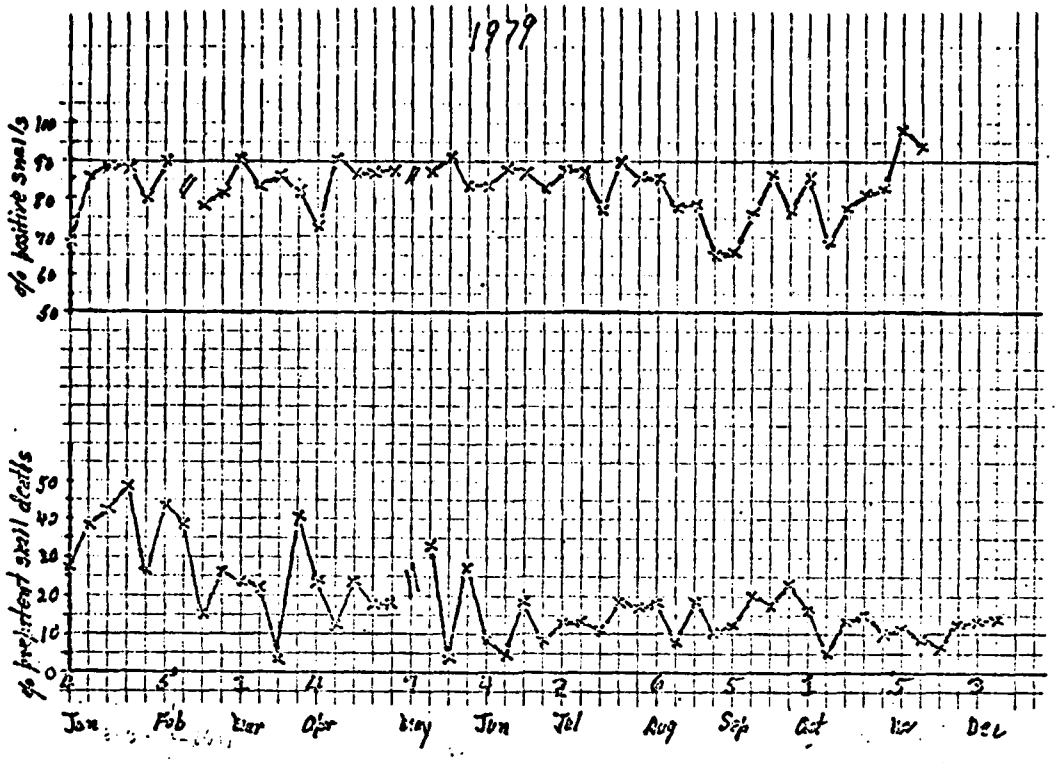

Figure 2.

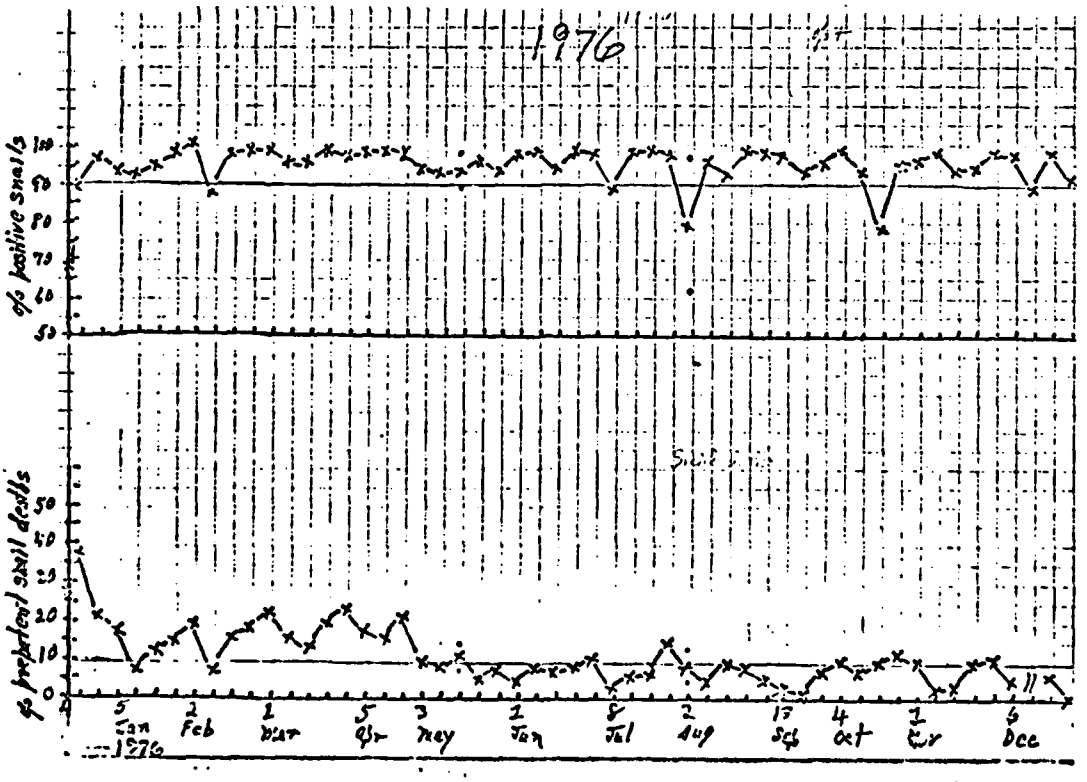

Figure 3.

period. There is no clear clue to the conditions responsible for periods of low infectivity (throughout 1979 compared with 1976) and high death rates (Jan through May 1979) in exposed snails. These periods appear to coincide with times when stock snails are laying very few to no eggs, perhaps indicating some inimical environmental condition for the stock snails, perhaps nutritional deficiency.

Discussion. The critical temperature for exposed snail maintenance (above 26 C) is necessary because a constant temperature below 25 C inhibits growth and development of the parasite in the snail and finally causes early termination of the infection.

A constant dark maintenance environment between collection periods eliminates any undesired light stimulus for the positively phototropic cercariae to emerge except during the cercarial collection period, so they accumulate in the snail host. Then when the infected snails are moved into a warmer bright light environment for cercarial collection, the accumulated cercariae emerge in the collecting beakers in large numbers. Transfer of the snails must be fast, however, to avoid emergence of the stimulated cercariae into the maintenance aquaria and thus their loss for collection. Cercariae are so sensitive to even small changes in the level of light intensity that an observable decrease in cercarial emergence per snail occurs with ageing of the light bulb. Note the higher cercarial collections from 9 May 1979 for about 3 months and again from 5 Nov 1979. The day before in each case the used fluorescent bulb in the cercarial collection chamber was replaced with a new one. Records of light intensity measured with a photovoltmeter are now being kept as the light bulb ages.

Conclusions. A high level of cercarial production (2500 to 3000 per snail) can be maintained if specific conditions are met. These conditions are concerned with stock snail health, age of the snails at exposure, level of exposure, and critical light and temperature.

II. THE INFECTIVE PROCESS - THE ROTIFER PROBLEM

Methodology. On each cercarial collection day, the snails were examined for rotifers which colonize the shells. Cercarial production per snail was compared in rotifer-infested and rotifer-clean snails. Mice were exposed to cercariae from each type of collection, and the percentage of penetrants/total cercariae and, 7 weeks later, the adult worm burdens, were established.

Rotifers were washed off the snails by jet spray, collected and the number per ml counted. The effect of rotifer emissions on cercariae was tested by incubating known numbers of rotifers in 10 ml of aged tap water for varying lengths of time, filtering the rotifers from the medium, and adding about 25 cercariae. The percentage of cercariae immobilized was recorded.

Results. Data in the following 3 figures and 1 table were reported also in Annual Report #4 of ONR Contract #N00014-76-C-0146 since funds from both contracts were used to support this work.

Numbers of cercariae emerging from rotifer-infested snails (R) were far below those from clean snails (C).

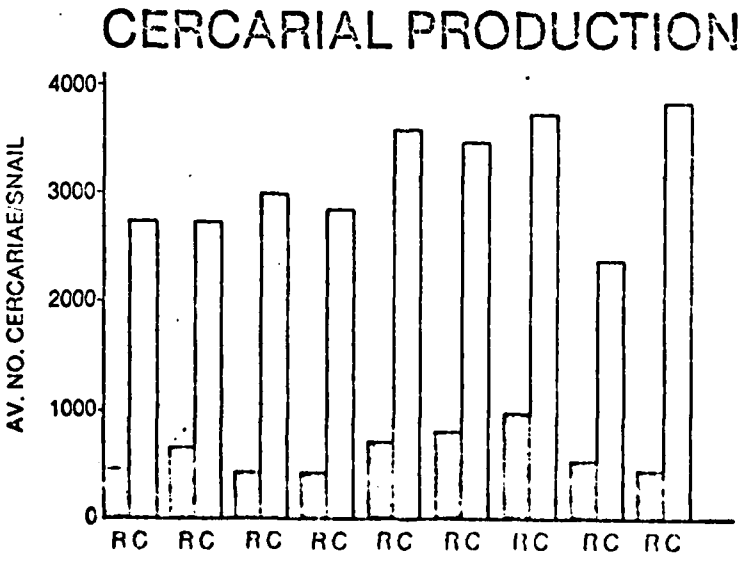

Figure 4.

The rotifer effect extended to the capacity of cercariae to penetrate skin and develop in mice. Penetration into mice of cercariae from rotifer-infested snails in the water in which they emerged, which contained snails with rotifers, was low (•-···•). The effect was reversible, since cercariae from rotifer-infested snails transferred to aged tap water (X———X) penetrated in almost as high percentage as cercariae from clean snails in emergence water (⊙···⊙). Penetration was highest by cercariae from clean snails transferred to aged tap water (⊗—⊗).

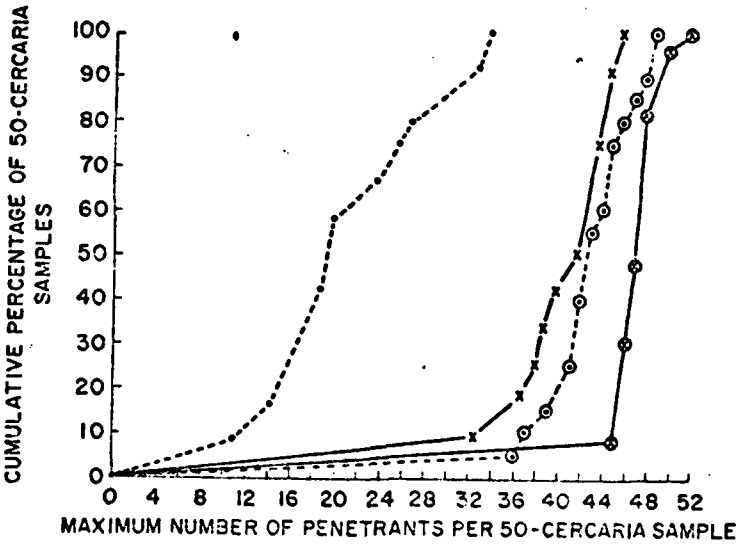

Figure 5.

Worm recovery was, of course, reduced also, as shown in the plot of worm burdens/mouse drawn according to the same scheme.

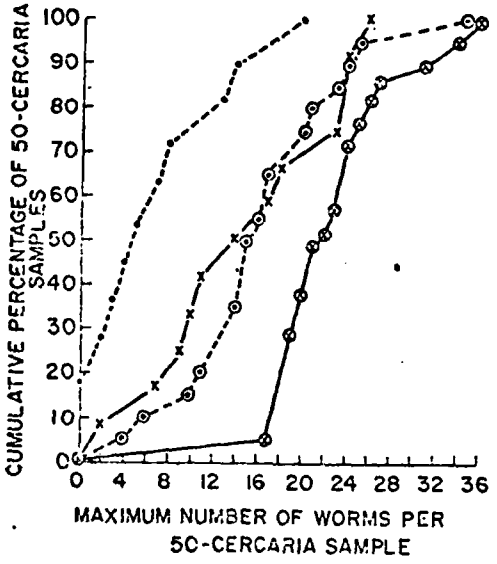

Figure 1.

Maturation of penetrants in mice (worms/penetrants), however, was not greatly affected, indicating the reversibility of the rotifer effect (Table 1).

Table 1. Summary of Effect of Rotifer Infestation of Snails on Schistosoma mansoni Cercariae. Data are presented as means and range of numbers or percentages. N=12 for unwashed cercariae; N=20 for washed cercariae.

	CERCARIAE FROM ROTIFER SNAILS		CERCARIAE FROM NON-ROTIFER SNAILS		
Cercarial Output (No. cerc/snail/day)	591	(276-1602)	3123	(2212-4080)	S*
Cercarial Penetration					
Unwashed** cercariae (%)	44	(22-69)	83	(66-92)	S
Washed cercariae (%)	87	(72-98)	95	(90-100)	S
Worm Burdens					
Unwashed cercariae (%)	14	(0-40)	31	(4-52)	S
Washed cercariae (%)	33	(8-70)	47	(34-72)	S
Schistosomule Maturation					
Unwashed cercariae (%)	31	(5-55)	37	(11-55)	NS
Washed cercariae (%)	40	(34-53)	49	(43-58)	NS

* S = Statistically significant; NS = not statistically significant.

** Unwashed means cercariae were used in water in which they had emerged from snails. Washed means cercariae in about 0.1 ml of emergence water were transferred to 7 ml of aged tap water for use.

Cercarial motility was affected not only in the presence of rotifers but also in rotifer-conditioned water, as demonstrated when cercariae from clean snails were pipetted into water in which rotifers had been incubated and then removed by filtration.

TABLE 2.

# Rotifers/ml water	Incubation Period (Days)	% Cercariae Immobilized within 5 min
300 ± 50	5	100
150 ± 25		100
75 ± 10		90
Control (aged tap water)		0

The minimal rotifer concentration and the least incubation time for conditioning water by rotifers has not yet been firmly established.

The rotifer factor is storage stable for at least 24 hours at room temperature (22 C). The rotifer-conditioned water tested above immobilized cercariae within 5 minutes when tested 24 hours after the end of the rotifer-conditioning period. It was heat labile, however, since cercariae exposed to it after it was heated at 100 C for 1 hour were not immobilized within 40 minutes.

Cercarial immobilization was reversible. If rotifer-conditioned water was replaced by aged tap water most of the cercariae recovered motility.

Infection of snails by miracidia was not influenced by rotifer infestation of the snail exposed.

DISCUSSION

Of the four aspects of the antischistosome effect of heavy rotifer infestation of infected snails thus far observed, the primary one was immobilization of cercariae. Immobilization was undoubtedly responsible for the inhibition of penetration of skin by cercariae from rotifer-contaminated snails and the resultant reduction in worm burdens in mice. The causal relationship between cercarial immobilization and the tremendous reduction in cercarial output by infected snails is more difficult to test. The effect may be directly on cercariae ready to emerge from the snail, but prevented from doing so by immobilization; or it may be on the snails and indirectly on their cercarial output. Even heavily rotifer-infested snails recovered their high cercarial output within three days after the rotifers were washed off.

Immobilization did not require the physical presence of rotifers. It occurred in rotifer-conditioned water from which the rotifers had been filtered out. The effect, therefore, was produced by some water-soluble emission of the rotifers. Quantitative studies in progress will indicate the number of rotifers per ml required to condition water to immobilize cercariae in any give time. The effect is seen first on the swimming movement of the cercarial tail, normal movement being replaced by spastic twitches. Affected cercariae sink slowly to the bottom of the observation tube, and finally do not respond to light changes by swimming as unaffected cercariae do.

That the rotifer effect on free-swimming cercariae is reversible is shown by the finding that immobilized washed cercariae recovered their motility, penetrated skin and developed into adults in numbers approximating those of washed non-rotifer

cercariae.

These findings bring up many questions. What is the source of the rotifer contamination? How can it be controlled? How does the rotifer factor act to reduce cercarial output?

While the adverse influence of rotifers on cercariae is undoubtedly of more practical interest in controlled laboratory conditions than in field situations, it may well be one of the natural controls along with certain Oligochaetes, such as Chaetogaster limnaei, Turbellaria, and miracidia-and cercaria-trapping water plants. If so, it may be a contributing factor to the low level of schistosome infections sometimes found in snails in endemic areas, and thus deserves elucidation and exploitation.

Conclusions. Rotifer infestation of snails in the laboratory is one of the most important inhibitory factors to collection of large numbers of motile infective cercariae.

III. MORPHOLOGICAL CHANGES AS CERCARIAE TRANSFORM TO SCHISTOSOMULES. ULTRASTRUCTURAL COMPARISON OF SCHISTOSOMULES PRODUCED IN VIVO AND IN VITRO.

Methodology. Schistosomules were produced by 5 methods: 1) in vivo by cercarial penetration of mouse ear skin in situ on anesthetized mice; 2) by cercarial penetration of dried rat epidermis in vitro; 3) without penetration, but stimulated to transform from cercariae after tail separation; 3) by shearing pressure while being forced through a 21 gauge hypodermic needle; 4) by subjection to centrifugation and temperature changes; and 5) by stirring in a modified Omnimixer. The organisms were cultured for 3, 6, 24, 48, 72, and 96 hours. Those generated in vivo remained in mouse ear tissue in situ, and those derived in vitro were kept in a culture

medium of Earle's salts containing lactalbumin hydrolysate, at 37 C in a CO_2 (5%) oven.

Verification of transformation to schistosomules by organisms derived from all 5 types of stimulation was made by testing water tolerance, glandular exhaustion, serodiagnostic envelope (CHR) formation and infectivity for mice.

For ultrastructural comparison, at the end of the culture schistosomules of each derivation were fixed by two methods: in 1% buffered osmic acid; and in 2.5% glutaraldehyde followed by post-fixation in 1% buffered osmic acid. Final fixation in both procedures was in 0.1% uranyl acetate for membrane preservation. Five organisms of each derivation type for each time period were examined in both transverse and longitudinal serial sections stained with uranyl acetate and lead citrate.

Results. Biological verification of the schistosomular status of the variously derived organisms is tabulated (Table 3).

Table 3. Level of Transformation to Schistosomules by the Variously Derived Organisms Below as Tested by the Parameters Listed

	In Vivo		In Vitro			
Parameter	Cerc		Rat Epidermis	Shear Pressure	Centrifuge/ Temperature	Omnimixed
Water Intolerance	No	<15 min	<1 hr	72 hr	<3 hr	>72 hr
Gland Exhaustion	No	<1 hr	3 hr	72 hr	Incomplete	72 hr
CHR Capability	100%	1%	7%	86%	88%	90%
Infectivity after Injection	Data Incomplete					

Organisms collected after penetration of dried rat epidermis and the in vivo schistosomules were essentially similar in transformation timing. In contrast, the organisms generated by the other three in vitro techniques transformed much more slowly. They differed among themselves only with regard to one parameter: the development of water intolerance.

As mentioned in a previous report, ultrastructural comparison of variously-derived schistosomules aged 30 minutes and 1 hour has been completed. A ms has been submitted to Experimental Parasitology. Against the background of cercarial fine structure, ultrastructural changes were described in organisms derived in vivo by skin penetration and in vitro by shear pressure. The same developmental pattern was observed in schistosomules of both derivations. In vitro schistosomules, however, developed more slowly, resembled cercariae more closely, and varied less among organisms than did in vivo schistosomules. The greatest morphological changes were observed in the 1-hr in vivo schistosomules. These were as follows: (1) in tegument, formation of transient microvilli, a hepatalaminate outer membrane and accented surface invaginations, loss of glycocalyx, movement outward of cyton vesicles via bridges, accumulation of multilaminate bodies around bridge openings; (2) in the anterior organ (oral sucker), movement of head gland vesicles via the ducts into tegument followed by collapse of the gland fundus, disappearance of the circumfundal cells and two large support cells, and the appearance in these areas of membranes and parenchymal cells; (3) secretion of the acetabular gland contents, collapse of the glands and replacement by membranes and parenchymal cells; (4) peristaltic activity of the digestive tract as shown by alternate areas of lumen constriction and dilation; (5) loss of bladder and contraction of

the small aboral collecting tubules; and (6) conversion of heterochromatic parenchymal cell nuclei to euchromatic. In contrast, the 1-hr in vitro shear schistosomules resembled 30-min in vivo schistosomules, retaining many cercarial features.

Detailed study of older organisms is incomplete. Three and 6 hour-old schistosomules derived in vivo and from in vitro shear pressure treatment have been sectioned and examined with the electron microscope. Older organisms have been fixed and embedded for sectioning. Through six hours, in vivo schistosomules had not developed appreciably beyond the 1 hour condition. In vitro shear pressure organisms cultured for 6 hours were similar in development to the one hour in vivo schistosomules; that is by 6 hours they had caught up with the schistosomules which had developed naturally in skin. Whether the in vitro organisms will experience the lag period characteristic of in vivo ones and thus continue to be delayed or whether they will maintain parallel development from the 6 hour stage has not yet been established.

Discussion. One objective of this work, namely, to establish whether or not artificially-derived (in vitro) organisms conform to the accepted criteria for true (in vivo) schistosomules, has been met. In vitro organisms showed the same developmental pattern as did in vivo postpenetration schistosomules and became bona fide schistosomules. On the other hand, in vitro-derived schistosomules developed more slowly. This was apparent both by biological testing and ultrastructurally. Using biological parameters, in vitro-derived schistosomules lagged about 3 days behind true schistosomules. As seen ultrastructurally also, they developed more slowly, lagging by at least 6 hours. Description

of fine structural differences among the artificially-produced schistosomules, a second objective, is in progress.

The "resting period" from 1 through 6 hours seen ultrastructurally in true schistosomules in skin confirmed this condition as it was described with the light microscope by Gordon and Griffiths. Such a developmental resting period may or may not occur in in vitro schistosomules. Detailed comparisons of organisms derived by the different currently-employed in vitro methods and ages through 4 or 5 days remain to be made.

Conclusions. Schistosomules produced by artificial means, in place of the normal method of skin penetration, are true schistosomules, as indicated by available tests. They develop more slowly than after skin penetration, however, and at different rates depending on the method used. Fine structural changes are similar but are delayed in artificially-derived schistosomules.

IV. ANTIGENICITY OF SECRETED CERCARIAL PREACETABULAR ENZYME.

A definition of the immunologic responses to discrete cercarial products in infected individuals may benefit diagnostic, epidemiologic, or protective immunity studies in schistosomiasis.

Methodology. Immunologic reactivity to cercarial preacetabular gland secretion was evaluated by lymphocyte proliferation of spleen cells obtained from:

(a) mice exposed to 500 ^{60}Co-irradiated cercariae, or

(b) mice infected with 30 normal cercariae. In mice in group (a) the response during developement of protective immunity to a challenge exposure was examined. In mice in group (b) the response developing during a patent infection was evaluated.

Results. Although the preacetabular gland components of 500 irradiated cercariae represented a much greater antigenic stimulus than that present in 30 normal cercariae, such stimulation failed to induce substantial specific reactivity in the exposed mice. Enhanced cellular reactivity to the cercarial secretion was seen only in those mice infected with 30 normal cercariae (Fig. 7). While the reactivity was sporadic, the increase in its magnitude generally coincided with the duration of the patent infection.

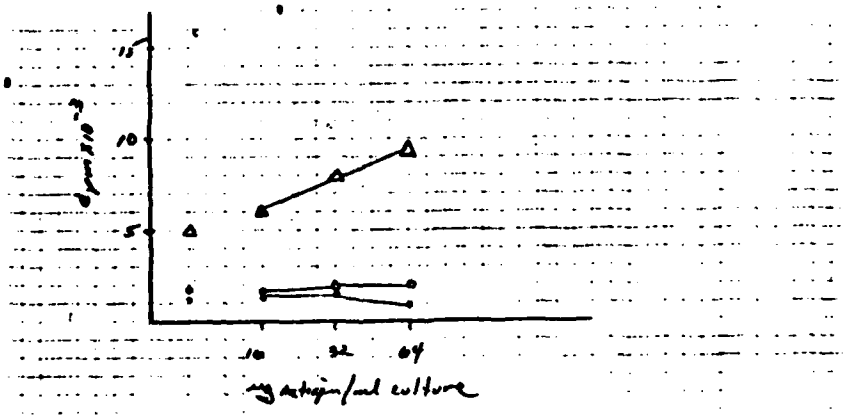

FIGURE 7. Uptake of ^{3}H-Thymidine by CBA/J spleen cells in response to differing concentrations of cercarial preacetabular gland material. Mice were uninfected (o—o), immunized 8 weeks previously with 500 ^{60}Co-irradiated cercariae (●—●), or infected 8 weeks previously with 30 normal cercariae (Δ—Δ).

Discussion. These results suggest that reactivity to the secretion products is dependent on a patent infection, and represents possible cross reactivity of the cercarial products with products of the adult worms or eggs. No differences in the response to the secretion were noted between a strain of mouse which develops protective immunity following exposure to irradiated cercariae (C57Bl/6) and one which does not (CBA).

Conclusions. Cercarial preacetabular gland secretions are antigenic, but response to them does not correlate with protective immunity. Enhanced cellular response developed only with a patent infection.

V. SEROLOGICAL CROSSREACTION AMONG PNEUMOCOCCAL POLYSACCHARIDES, PLANT POLYSACCHARIDES AND SEVERAL PARASITES INCLUDING SCHISTOSOMES. (Dr. Prescott, NIH Supplement).

Methodology. Polysaccharides were isolated from 10 pneumococcal types of Pleurotes ostreatus and Lentinus edodes. The pneumococcal organisms were cultivated in Todd-Hewitt medium at 37 C for 18 hours and the fungi for 4 weeks. Cells were removed by centrifugation for the preparation of 200 ml of heat-killed formalin-treated bacterial suspensions. Organisms were injected into rabbits for preparation of specific antisera which were tested for their reactivity against mouse lymphocytes.

Polysaccharides prepared by the Prescott calcium phosphate procedure were purified by alcohol reprecipitation and filtration through Seitz filterpads. Final precipitate was collected by centrifugation, dissolved in distilled water, refiltered through Seitz filterpads, dialyzed for 48 hours against at least 10 volumes of distilled water, and the solutions lyophilized.

Purity of the samples was analyzed by the following tests: total nitrogen, phosphorus, inorganic residue, nucleic acid and optical rotation. Serological testing was with the precipitin reaction and Ouchterlony test with specific antisera.

Results. On the polysaccharides isolated, Hopkins-Cole, Xanthoprolein, Millons and ninhydrin tests for protein were negative. The iodine test for glycogen was negative. The Molisch test for carbohydrate was positive in all samples tested. The pneumococcal polysaccharides isolated gave high precipitin titers and positive Ouchterlony tests. The fungal polysaccharides cross-reacted with Type III pneumococcal antiserum in the precipitin reaction with titers of 1:640,000 and gave lines in the Ouchterlony test that were confluent with Type III pneumococcal polysaccharides.

Discussion. Reactivity of the plant polysaccharides in the precipitin and Ouchterlony testing with Type III pneumococcal antiserum and the ability to elicit antibody formation in spleen of Balb/c mice may be explained by the presence of side chains of cellobiuronic acid attached to a main chain or nucleus composed of glucose, galactose and mannose units.

SIGNIFICANT ACCOMPLISHMENTS

1. The finding that fluctuations in cercarial production and in exposed snail deaths are dependent on specific controllable laboratory conditions for maintenance of the snails and for cercarial collections has meant that variability in these parameters has been reduced. As a result, any schistosome laboratory which is so motivated can produce large numbers of infective cercariae regularly.

2. One of the critical conditions above is the inhibitory effect of rotifer-colonization of snails on cercarial output and motility. The effect has been shown to be mediated by emissions from the rotifers. These emissions are water soluble, heat labile and relatively storage stable. It is possible that chemical analysis of the rotifer factor may provide for rotifer control in the laboratory on one hand and for a field control measure against schistosomes on the other hand.

3. The question of acceptability of artificially-produced schitosomules as compared with the normal postpenetration schistosomules has been answered by biological testing and fine structural comparisons of organisms produced by the various methods in use. Artificially-derived schistosomules underwent the same developmental changes as true schistosomules, albeit more slowly. Insofar as can be tested, those produced artificially are acceptable for experimental use.

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